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Inflammatory response of LPS-hyporesponsive and LPS-responsive mice to challenge with gram-negative bacteria *Salmonella typhimurium* and *Klebsiella pneumoniae*

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Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFRRI SR82-18	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) INFLAMMATORY RESPONSE OF LPS-HYPORESPONSIVE AND LPS-RESPONSIVE MICE TO CHALLENGE WITH GRAM-NEGATIVE BACTERIA S. TYPHIMURIUM AND K. PNEUMONIAE		5. TYPE OF REPORT & PERIOD COVERED 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) T. J. MacVittie, A. D. O'Brien,* R. I. Walker,† and S. R. Weinberg (cont. on reverse)		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Armed Forces Radiobiology Research Institute (AFRRI) Defense Nuclear Agency Bethesda, Maryland 20814		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NWED QAXM MJ 00029
11. CONTROLLING OFFICE NAME AND ADDRESS Director Defense Nuclear Agency (DNA) Washington, DC 20305		12. REPORT DATE November 1982 13. NUMBER OF PAGES 15
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

In: Macrophages and Natural Killer Cells. S. J. Normann and E. Sorkin, eds. Plenum Publishing Corporation, New York, 1982, pp. 325-334.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The endotoxin(LPS)-resistant strain mice, C3H/HeJ (HeJ) are conversely and markedly sensitive to at least two strains of gram-negative bacteria, S. typhimurium (S.t.) and K. pneumoniae (K.p.), relative to their endotoxin-sensitive paired C3H/HeN (HeN) strain mice. The 100% lethal dose (LD100) of S.t. for the HeJ mice was 10^1 organisms whereas the LPS-sensitive HeN strains required 10^7 organisms. Relative LD100 for infection with K.p. was similar to that noted for S.t.

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

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20. ABSTRACT (continued)

In this report we examined the peritoneal nucleated cell response (TNC) and cellular composition to infection with S.t. and K.p. Peak TNC responses were similar, ~ 3-4 times the resident value, irrespective of mouse or bacterial strain. However, cellular composition of respective peritoneal exudates showed significant differences between LPS-sensitive and LPS-resistant strains of mice. It was further seen that the differential cellular response also depended on bacterial strain, evident with S.t. and K.p.

Cellular responses of LPS-sensitive HeN and C57BL/6 strain mice were predominantly mononuclear and macrophage in composition. Macrophages and lymphocytes increased to peak values ~3-4 times resident values, 80%-90% of TNC. Neutrophils (PMN) were only 1%-5% of TNC, but showed a predominant biphasic response. In marked contrast, HeJ responded with a significant PMN influx, increasing 200-300 times resident values and 60%-70% of TNC just before death of the animal. Macrophages increased only 1.7 times resident values, and lymphocytes did not increase above resident values. This differential response was not observed in S.t.-sensitive C57BL/6 mice. Susceptibility of inbred mice to infection appears to be controlled by host genetic factors as well as mechanisms of bacterial pathogenesis.

From: MACROPHAGES AND NATURAL KILLER CELLS
Edited by Siegfried S. Wagner and Ernst Sorkin
(Plenum Publishing Corporation, 1982)

INFLAMMATORY RESPONSE OF LPS-HYPORESPONSIVE AND LPS-RESPONSIVE MICE TO CHALLENGE WITH GRAM-NEGATIVE BACTERIA SALMONELLA TYPHIMURIUM AND KLEBSIELLA PNEUMONIAE

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INTRODUCTION

The murine response to lipopolysaccharide (LPS) or endotoxin is determined by the allelic form of the Lps gene carried by the host (1,2). Mice that are homozygous for the defective Lps^d allele, such as C3H/HeJ (HeJ) animals, respond to only high doses of endotoxin, whereas mice that are homozygous or heterozygous for the normal Lps^n allele, e.g., C3H/HeN (HeN) mice, react to low-dose challenge. Thus, HeJ mice are insensitive to quantities of LPS that elicit mitogenic, inflammatory, hemopoietic, or lethal effects in HeN mice (3–10). Furthermore, the nature of the cellular influx into the peritoneum of LPS-inoculated HeN and HeJ mice differs. Low doses of LPS (1–10 μg) induce an early polymorphonuclear (PMN) increase, followed by a rapid rise in macrophages and macrophage colony-forming cells in the HeJ peritoneal cavity (7,11,12). By contrast, a relatively small PMN infiltrate is evident in the HeN peritoneal inflammatory response, and the onset of the macrophage influx is delayed compared to the HeJ response.

The purpose of this investigation was to determine whether the inflammatory responses of HeJ and HeN mice to killed or viable gram-negative bacteria mimicked those seen after administration of LPS. This question was of particular interest because endotoxin-hypore-

sponsive HeJ mice are very susceptible to infection with the gram-negative bacteria Salmonella typhimurium (13-15) and Klebsiella pneumoniae (16), whereas endotoxin-sensitive HeN mice are relatively resistant to these LPS-containing microbes. In addition, O'Brien et al. (15) have found that the gene that controls the response of the HeJ mice to S. typhimurium is closely linked to or the same as the single gene defect that controls their response to LPS.

METHODS AND MATERIALS

Peritoneal cell suspensions were obtained by lavage from 8-to-12-week-old male or female mice of the strains C3H/HeN (Charles River, Wilmington, MA) and C3H/HeJ (Jackson Lab, Bar Harbor, ME). The animals were maintained on a 12-hour light-dark cycle. Wayne Lab-Blox and acidified (pH 2.5) water were available ad libitum. All mice were acclimated to laboratory conditions for 2 weeks before experimental treatment. During this time, the mice were examined and found to be free of lesions of murine pneumonia complex and of oropharyngeal Pseudomonas sp. Lavage was accomplished by i.p injection and subsequent withdrawal of 4 ml of Hanks' Balanced Salt Solution (calcium- and magnesium-free). Total and differential cell counts were performed on samples of exudate cells. Smears of cells were prepared with the use of a cytospin centrifuge (Shandon Southern Institute, Ltd., Sewickley, PA), air-dried, and stained with a Wright-Giemsa solution.

Peritoneal exudates were induced by injection of (a) 10 μg of E. coli 055.B5 lipopolysaccharide-W (LPS-W) (List Biological Labs, Campbell, CA), (b) 1.5 mg (wet weight) of formalin-killed Klebsiella pneumoniae, or (c) viable S. typhimurium or K. pneumoniae at doses of 10 organisms for HeJ mice or 1000 organisms for HeN animals. The S. typhimurium (strain TML) (17) was grown overnight at 37°C with shaking in Penassay broth (Difco Labs), whereas the encapsulated K. pneumoniae strain was cultured in brain heart infusion (BHI) broth (Difco). Bacterial challenge doses were prepared in sterile pyrogen-free saline and the actual inoculum size verified by plate count on BHI (K. pneumoniae) or tryptic soy agar (S. typhimurium). For killed preparations of K. pneumoniae, 18-hour broth cultures were treated with 0.75% formalin for 2 hours at room temperature with constant stirring. An aliquot of cells was incubated in BHI broth to determine sterility of the killed-cell suspension.

The 50% lethal dose (LD_{50}) of an agent for mice was determined by the method of Reed and Muench (18). Groups of five age-matched mice were inoculated i.p. with graded doses of each substance, and deaths were recorded daily for 28 days.

RESULTS

Lethal Dose 50 Values of LPS or Bacteria for Mice

HeJ strain mice were significantly more resistant to LPS and killed K. pneumoniae than were mice of the HeN strain. The LD_{50} of LPS or killed K. pneumoniae for HeJ mice was 2000 μg and 65 mg, respectively, whereas only 150 μg of LPS or 7.5 mg of killed bacteria was sufficient to kill 50% of HeN mice (Table 1). Conversely, all HeJ mice succumbed to S. typhimurium or K. pneumoniae challenge with <10 organisms, but the LD_{50} of these microbes for endotoxin-sensitive HeN animals was 2,000.

Total Peritoneal Cell Influx after Challenge with Bacteria or LPS

As shown in Table 2, the total number of resident peritoneal nucleated cells was equivalent in the HeN and HeJ mice (range = 3.8 to 4.7 x 10^6). When mice were given LPS-W or viable K. pneumoniae or S. typhimurium, the magnitude of the total cellular influx was also similar for HeN and HeJ mice. However, administration of killed K. pneumoniae elicited a significantly greater ($p < 0.01$) response in HeJ than in HeN animals.

Differential Inflammatory Response of C3H/HeN and C3H/HeJ Strain Mice

The inflammatory response of the HeJ strain mouse to LPS was characterized by an early PMN response followed by a subsequent and predominant monocyte-macrophage influx that peaked at day 4 after

Table 1. Comparative Lethality of S. typhimurium, K. pneumoniae, LPS-W, and killed K. pneumoniae for C3H/HeN and C3H/HeJ Strain Mice

Mouse Strain	LPS/W	Killed K. pneumoniae	S. typhimurium	K. pneumoniae
C3H/HeN	150 μg	7.5 mg	1,000-10,000	1,000-10,000
C3H/HeJ	2000 μg	65.0 mg	10	10

Comparative lethality is reported as the LD_{50} dose by weight for LPS-W (obtained from S. typhimurium) and formalin killed K. pneumoniae (wet weight) and by numbers of organisms for viable K. pneumoniae and S. typhimurium injected intraperitoneally.

Table 2. Peak Peritoneal Nucleated Cell Influx in C3H/HeN and C3H/HeJ Strain Mice at Day 4 after Challenge

Challenge	Resident Cells	Day-4 Cells
LPS-W:		
HeN mice	4.2±0.5	10.5±1.5
HeJ mice	4.4±0.7	13.0±2.0
Killed K. pneumoniae:		
HeN mice	3.8±0.6	10.3±1.2
HeJ mice	4.2±0.8	15.3±1.8
K. pneumoniae:		
HeN mice	4.7±0.6	15.0±2.1
HeJ mice	4.3±0.5	13.0±1.7
S. typhimurium:		
HeN mice	4.1±0.7	20.2±2.2
HeJ mice	4.2±0.7	17.1±2.1

Mice were challenged with 10 μg of LPS-W; 1.5 mg of formalin killed K. pneumoniae; 10 organisms of K. pneumoniae or S. typhimurium into HeJ or 1000 organism into HeN mice. The data are reported as mean total nucleated cells ± standard error x 10^6.

injection (Figure 1). Lymphocytes also increased twofold over resident values. Influxes of all three cell types in the HeN mice were delayed relative to the HeJ mice, but HeJ and HeN mice attained equivalent numbers of mononuclear cells by 72-96 hours after injection. These results confirmed the findings of Sultzer and Goodman (11) and Moeller et al. (7).

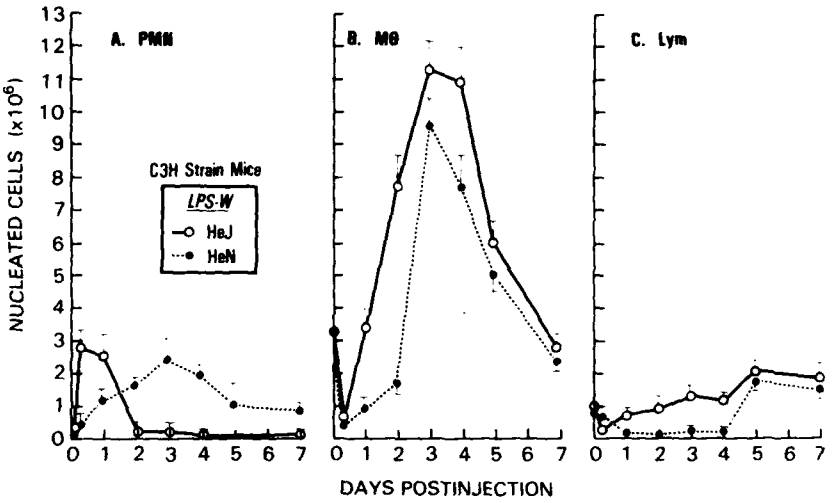

Fig. 1. Peritoneal cellular influx of (A) polymorphonuclear leukocytes (PMN), (B) macrophages (MΘ), and (C) lymphocytes (Lym) in C3H/HeN (●) and C3H/HeJ (○) mice injected with 10 μg of LPS-W. Mean values (±SEM) of four replicate experiments.

Similar responses were observed after injection of killed K. pneumoniae. Both murine strains showed a predominantly mononuclear influx, although the HeJ mice responded to a significantly greater degree than did HeN mice (Figure 2).

Markedly different responses were observed following infection of HeJ or HeN mice with either live K. pneumoniae (Figure 3) or S. typhimurium (Figure 4). The HeN mouse showed an early influx of PMNs followed by a rise in mononuclear cells. In contrast to the cellular response of HeN mice to LPS-W and killed K. pneumoniae, the mononuclear fraction elicited in response to live bacteria contained a greater share of lymphocytes (Figures 3 and 4). The HeJ mouse also responded with a significant PMN influx, but unlike the HeN mouse, the influx continued to increase until death of the HeJ animal within 5 days after infection with either K. pneumoniae or S. typhimurium (Figures 3 and 4). The mononuclear response, although initiated in the HeJ mouse, failed to maintain itself as the infection progressed.

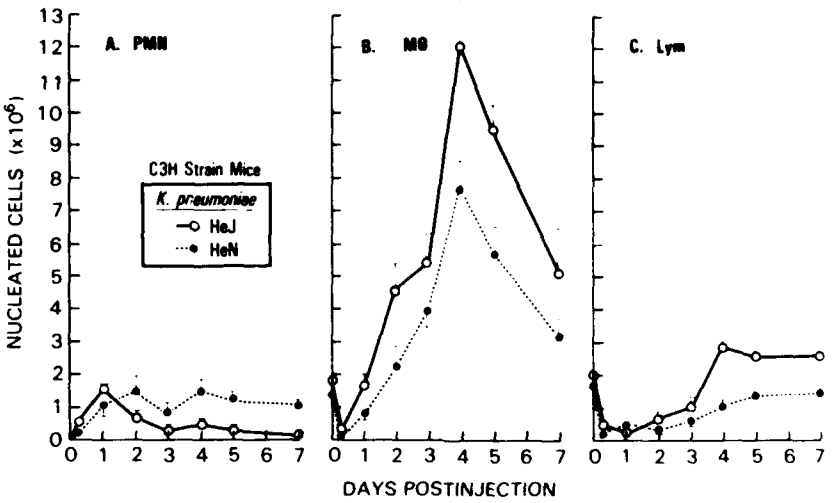

Fig. 2. Peritoneal cellular influx of (A) polymorphonuclear leukocytes (PMN), (B) macrophages (MΦ), and (C) lymphocytes (Lym) in C3H/HeN (●) and C3H/HeJ (o) mice injected with 1.5 mg (wet weight) killed K. pneumoniae. Mean values (± SEM) of four replicate experiments.

DISCUSSION

In this investigation, the inflammatory responses of endotoxin-hyporesponsive C3H/HeJ mice and endotoxin-responsive C3H/HeN mice were compared after the animals had been given an LPS preparation, killed K. pneumoniae as a source of endotoxin, or viable LPS-containing S. typhimurium or K. pneumoniae. Three parameters of the response were examined: the quantitative cellular response, the types of cells involved, and the kinetics of the cellular influx.

The data indicated that in response to LPS or killed K. pneumoniae as a source of endotoxin, the HeJ mouse responded with a predominantly mononuclear influx that was equivalent to or greater than that of the endotoxin-responsive HeN strain. Thus, the HeJ mouse was sensitive to the presence of endotoxin as a soluble molecule or as formalin-killed gram-negative K. pneumoniae. However, infection of the HeJ mice with S. typhimurium or K. pneumoniae produced an inflammatory response composed predominantly of PMNs rather than mononuclear cells as was seen in resistant HeN mice. Since the total cellular influx was equivalent between the two C3H strains, the PMN

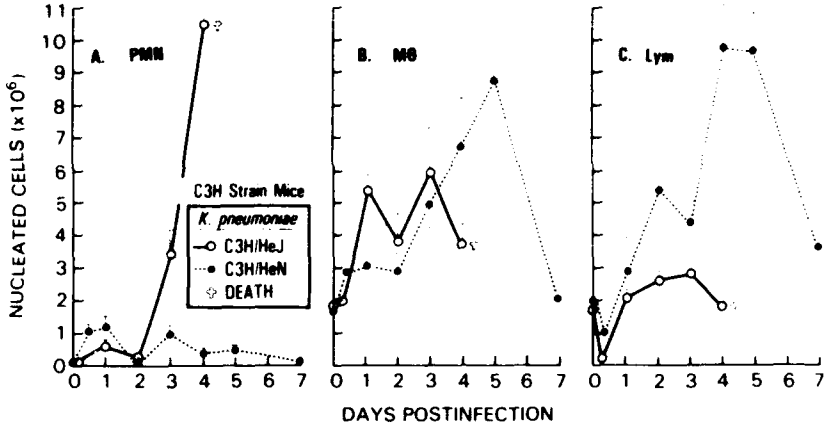

Fig. 3. Peritoneal cellular influx of (A) polymorphonuclear leukocytes (PMN), (B) macrophages (MΦ), and (C) lymphocytes (Lym) in C3H/HeN (●) and C3H/HeJ (o) mice infected with 1,000 or 10 K. pneumoniae, respectively. Mean values (± SEM) of five replicate experiments.

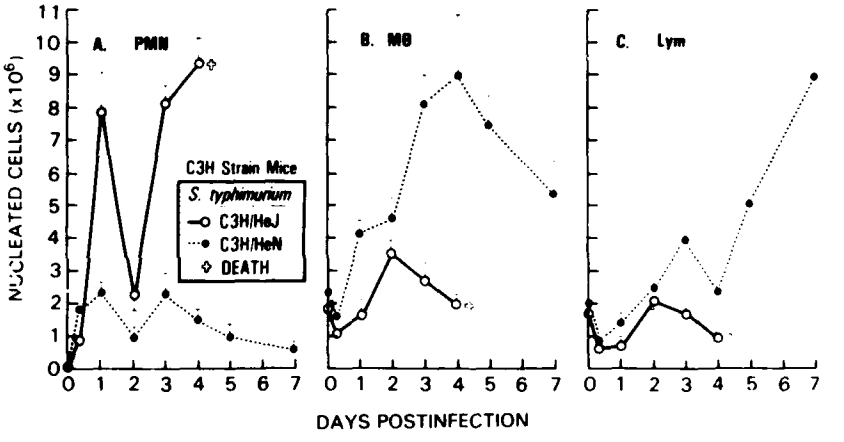

Fig. 4. Peritoneal cellular influx of (A) polymorphonuclear leukocytes (PMN), (B) macrophages (MΦ), and (C) lymphocytes (Lym) in C3H/HeN (●) and C3H/HeJ (o) mice infected with 1,000 or 10 S. typhimurium, respectively. Mean values (± SEM) of five replicate experiments.

influx represented a marked shift away from the normal immigration of mononuclear cells. Jerrels and Osterman (19) have recently described a similar PMN response in a strain of C3H mice susceptible to infection with Rickettsia tsutsugamushi. Thus, the phenotypic expression of the Lps^d allele, as measured by the nature of the peritoneal cell infiltrate, is markedly different when endotoxin is presented to the hyporesponsive host on viable, replicating gram-negative bacteria rather than on killed microbes or as an extract.

Two lines of evidence suggest that the failure of endotoxin-hyporesponsive HeJ mice to mount a normal macrophage reaction to S. typhimurium infection may be directly related to their innate susceptibility to this microbe. First, macrophages, not PMNs, are the effector cells in murine resistance to salmonellosis (20). Second, recent studies by O'Brien et al. (21) indicate that for S. typhimurium, susceptibility of Lps^d animals is a consequence of a macrophage abnormality. That such a macrophage dysfunction may be quantitative as well as qualitative is suggested by the data presented herein.

Acknowledgement

Supported by Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit MJ 00029. The views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred. The authors gratefully acknowledge the excellent technical assistance of James L. Atkinson, Brenda Watkins, and Daniel P. Dodgen. We also thank Junith A. Van Deusen and Doris M. Pateros for editing and preparing this manuscript.

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DISCUSSION

GINSBERG: When you use LPS, it is usually either commercial material or that produced yourself by phenol extraction or some similar method. Is this the real LPS which is released in vivo? What do we know about the mechanisms by which LPS is released from gram negative organisms in vivo? Is it by autolysis of the gram negative organism? Is LPS attacked by lysozomal enzymes or by antibody and complement on the surface of the cell?

The dichotomy you observed between resistance or susceptibility to commercial LPS versus the whole, killed organism raises another question. Is it possible that some humoral substance or some enzymes are missing in vitro which cleave the LPS off the bacterial cells in vivo? The reason for raising this question comes from work done in our laboratory recently showing that LPS can be obtained from gram negative organisms by lysozomal factors from granulocytes. If you kill the microbe by antibiotics, heat or ultraviolet radiation, you no longer release the natural LPS by treatment with lysozomal factors from granulocytes. I suggest that you look at the LPS released in vivo by natural mechanisms. Such probably would shed more light on the various genetic groups of mice that you are dealing with.

MacVITTIE: Thank you for that comment. I agree with you 100%. We

did perform several studies using LPS-W (Westphal) and LPS-B (Boivin) as well as a purified LPS but we wondered if the information had relevance to the whole viable organism? In other words, how does the animal see this organism and what are the interacting factors?

GINSBERG: There are certain strains of gram negative mutants, which are either very susceptible or very resistant to the effect of antibody and complement mediated bacteriolysis. Since bacteriolysis may be the origin of the genuine LPS, it would be prudent to examine highly resistant, serum resistant and serum susceptible strains of infectious organisms.